

# Binding between the Neural Cell Adhesion Molecules Axonin-1 and Nr-CAM/Bravo Is Involved in Neuron–Glia Interaction

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**Abstract.** Neural cell adhesion molecules of the immunoglobulin superfamily mediate cellular interactions via homophilic binding to identical molecules and heterophilic binding to other family members or structurally unrelated cell-surface glycoproteins. Here we report on an interaction between axonin-1 and Nr-CAM/Bravo. In search for novel ligands of axonin-1, fluorescent polystyrene microspheres conjugated with axonin-1 were found to bind to peripheral glial cells from dorsal root ganglia. By antibody blockage experiments

an axonin-1 receptor on the glial cells was identified as Nr-CAM. The specificity of the interaction was confirmed with binding studies using purified axonin-1 and Nr-CAM. In cultures of dissociated dorsal root ganglia antibodies against axonin-1 and Nr-CAM perturbed the formation of contacts between neurites and peripheral glial cells. Together, these results implicate a binding between axonin-1 of the neuritic and Nr-CAM of the glial cell membrane in the early phase of axon ensheathment in the peripheral nervous system.

THE development of the nervous system involves distinct cellular processes, including neuronal cell migration, axon outgrowth and guidance, synapse formation, and neuron–glia interactions such as myelination. Each of these activities requires precise cell–cell and cell–extracellular matrix (ECM)<sup>1</sup> interactions, which are mediated by cell-surface glycoproteins and ECM components. Cell-surface glycoproteins with a function in cell adhesion during the development of the nervous system can be divided into three major structural classes: (a) integrins, which bind to various ECM components (Reichardt and Tomaselli, 1991; Hynes, 1992); (b) cadherins, which mediate cell–cell adhesion in a calcium-dependent manner (Takeichi, 1991); and (c) cell adhesion molecules (CAMs) of the Ig superfamily, which are involved in calcium-independent homophilic binding to identical molecules and heterophilic binding to other family members or structurally unrelated molecules (Jessell, 1988; Grumet, 1991; Brummendorf and Rathjen, 1993).

Here we report on a novel interaction between the neural CAMs axonin-1 and Nr-CAM/Bravo. Both belong to a

subcategory of neural Ig superfamily molecules that is formed by proteins containing both Ig- and fibronectin type III (FNIII)-like repeats and therefore are termed Ig/FNIII-like proteins (Sonderegger and Rathjen, 1992; Brummendorf and Rathjen, 1993). The chicken glycoprotein axonin-1 (Zuellig et al., 1992), as its rat homologue TAG-1 (Furley et al., 1990), consists of six Ig-like and four FNIII-like domains and is membrane bound via a glycosyl-phosphatidylinositol anchor. In vitro perturbation experiments with antibodies revealed that axonin-1 is involved in fasciculation of neurites (Ruegg et al., 1989b). Upon presentation as a substratum to cultured dorsal root ganglia (DRG) neurons, axonin-1 strongly promotes neurite outgrowth (Stoeckli et al., 1991). The temporal and spatial expression pattern of axonin-1 in the chicken (Ruegg et al., 1989b; Halfter et al., 1994; Giger et al., 1995), as well as of its homologue TAG-1 in the rat (Dodd et al., 1988; Furley et al., 1990) and the mouse (Wolfer et al., 1994), is consistent with these functions in vitro. Two distinct binding activities of axonin-1 have been demonstrated to date: (1) A heterophilic interaction with the Ig/FNIII-like protein Ng-CAM has been reported to be involved in neurite outgrowth (Kuhn et al., 1991); and (2) a homophilic interaction is observed if axonin-1 is heterologously expressed at the surface of myeloma cells (Rader et al., 1993), but not if purified axonin-1 is covalently bound to polystyrene microspheres (Kuhn et al., 1991). The functional role of this homophilic interaction has not yet been characterized.

Nr-CAM/Bravo was initially discovered on developing

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1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; DRG, dorsal root ganglia; ECM, extracellular matrix; Fab, fragment with an antigen-binding site; FNIII, fibronectin type III; RT, room temperature.

optic fibers in the chicken retina and termed Bravo (de la Rosa et al., 1990). Its cDNA structure was subsequently reported under the name of Nr-CAM (Grumet et al., 1991) and Bravo/Nr-CAM (Kayyem et al., 1992b), respectively. The designation Nr-CAM, which is used more widely in the literature, will be used in this report. Nr-CAM is composed of six Ig-like and five FNIII-like domains, a transmembrane segment, and a cytoplasmic domain of ~110 amino acid residues. The expression pattern of Nr-CAM and *in vitro* studies of its function suggest that Nr-CAM is involved in neurite outgrowth and fasciculation (de la Rosa et al., 1990; Grumet et al., 1991; Krushel et al., 1993; Morales et al., 1993). Two different binding functions for Nr-CAM have been identified so far: a homophilic binding is thought to mediate neurite fasciculation (Mauro et al., 1992), and a heterophilic interaction with the Ig/FNIII-like glycoprotein F11 is involved in neurite outgrowth of tectal cells on immobilized F11 (Morales et al., 1993). A second heterophilic-binding activity of Nr-CAM to a yet unidentified receptor on fibroblasts has also been reported; in contrast to the other two interactions it was found to be divalent cation dependent (Mauro et al., 1992). In search for novel ligands of axonin-1 using a binding assay with axonin-1 conjugated to fluorescent polystyrene microspheres (Covaspheres), we have found binding sites for axonin-1 on cultured peripheral glial cells from chicken DRG. Antibody perturbation studies identified Nr-CAM as a receptor for axonin-1 on these cells. *In vitro* application of antibodies against axonin-1 or Nr-CAM perturbed the formation of close contacts between DRG neurites and peripheral glial cells, suggesting a role for the interaction between axonin-1 and Nr-CAM in the early phase of axon ensheathment.

## Materials and Methods

### Proteins and Antibodies

Axonin-1 was purified from the ocular vitreous fluid of 14-d-old chicken embryos by a four-step chromatographic procedure using fast performance liquid chromatography (FPLC) (Pharmacia Biotechnology, Dübendorf, Switzerland) (Ruegg et al., 1989a). Nr-CAM/Bravo was immunoaffinity purified from detergent solubilized brain membranes of posthatching day 1 chickens as previously reported (de la Rosa et al., 1990), with the following modifications: brains were homogenized in 5 ml each of extraction buffer (10 mM Hepes, pH 7.5, 140 mM NaCl, 4 mM EDTA, 10  $\mu$ l/ml aprotinin, 0.1 mg/ml PMSF, 50  $\mu$ g/ml soybean trypsin inhibitor, 2 mg/ml iodine acetamide, 1  $\mu$ M leupeptin [all protease inhibitors were from Sigma Chemical Co., St. Louis, MO], 1 mg/ml RNase, 1 mg/ml DNase, 1% [wt/vol] Zwittergent 3-14 [Calbiochem, Frankfurt, Germany], and 1% [wt/vol] NP-40 [Calbiochem]). The homogenate was cleared by a 3,000-g and a 100,000-g centrifugation step (30 min each) and the supernatant was loaded on a column containing Affi-Gel 10 (Bio-Rad Laboratories, Munich, Germany) coupled to the monoclonal anti-Nr-CAM/Bravo antibody 2B3 (de la Rosa et al., 1990; Kayyem et al., 1992a) generated by Dr. Enrique J. de la Rosa (Centro de Investigaciones Biológicas CSIC, Madrid, Spain). For the elution, the detergents were replaced by 0.2% (wt/vol) deoxycholate (Sigma Chemical Co.). Purified Nr-CAM was subsequently dialyzed against PBS containing 0.2% (wt/vol) deoxycholate. Ng-CAM was isolated from brain membranes of 14-d-old chicken embryos by immunoaffinity chromatography, using the mAb 12-I4E-311 kindly provided by Dr. Fritz G. Rathjen (Rathjen et al., 1987). The purity of the isolated proteins was tested by SDS-PAGE and checked for cross-contamination by immunoblotting using the polyclonal antisera described below. No cross-contamination of purified axonin-1, Nr-CAM, or Ng-CAM was observed (data not shown). BSA was purchased from GIBCO-BRL Life Technologies (Basel, Switzerland).

Antisera against axonin-1 and Ng-CAM were raised in goats (Ruegg et al., 1989a), antiserum against Nr-CAM was raised in rabbits essentially as described for the SC1/DMGRASP antiserum (Pollerberg and Mack, 1994). Polyclonal antibodies were tested for cross-reactivity by immunoblotting and dot-blot assay using purified proteins. No cross-reactivity of the antisera was detected (data not shown). IgG from goat antisera and goat preimmune serum was purified by affinity chromatography on protein G-Sepharose (Pharmacia Biotechnology). IgG from rabbit antiserum was purified by affinity chromatography on protein A-Sepharose (Pharmacia Biotechnology). Fragments with an antigen-binding site (Fab) were prepared by proteolytic digestion with papain (Sigma Chemical Co.) followed by chromatographic purification on a Mono S column (Pharmacia Biotechnology) as previously described (Ruegg et al., 1989b). For antibody perturbation studies, Fab fragments were dialyzed against PBS and concentrated by ultrafiltration (Ultrafree-20; Millipore Corp., Bedford, MA).

### Covalent Coupling of Proteins to Fluorescent Microspheres

Purified axonin-1, Nr-CAM, Ng-CAM, and control proteins, such as BSA and nonimmune IgG, were covalently coupled to TRITC- or FITC-labeled fluorescent polystyrene microspheres (Covaspheres; nominal diameter 0.5  $\mu$ m; Duke Scientific Corp., Palo Alto, CA) as previously described (Kuhn et al., 1991). In brief, 50  $\mu$ g of each protein was added to  $10^{10}$  Covaspheres in 1 ml PBS for 1 h at 37°C (PBS containing 0.2% deoxycholate in the case of Nr-CAM; PBS containing 0.5% CHAPS in the case of Ng-CAM). Unbound protein was removed after sedimentation of the beads (16,000 g, 10 min, 4°C). The Covaspheres were resuspended in PBS containing 5 mg/ml BSA and 10 mM sodium azide and sonicated for 2 min in a bath sonicator (Elma; Singen, Germany). After incubation for 30 min at room temperature (RT) and a second centrifugation, the beads were resuspended in 100  $\mu$ l of the same buffer and stored at 4°C. To determine the coupling yield serial dilutions of the initial protein solution and the unbound protein from the supernatant of the coupling reaction were subjected to SDS-PAGE. The protein bands were visualized by silver staining. Protein bands with identical intensities were identified, and from their dilution factor the ratio between the coupled and uncoupled protein was calculated. In all coupling reactions, a similar yield of ~50% was obtained.

### Flow Cytometric Analysis of the Aggregation of Protein-conjugated Covaspheres

Protein-conjugated Covaspheres were coincubated in dual combinations for 1 h at RT as described by Kuhn et al. (1991). The incubation mixture contained  $10^{10}$ /ml Covaspheres of each species in a final vol of 20  $\mu$ l PBS containing 5 mg/ml BSA and 10 mM sodium azide. Antibody perturbation of Covasphere aggregation was carried out by preincubation of one of the two samples with Fab fragments of polyclonal IgG at a concentration of 500  $\mu$ g/ml in PBS for 2 h at RT. Unbound antibodies were removed by two washes with PBS. Samples taken from the incubation mixture were analyzed for aggregate formation using both a fluorescence microscope (after a 1:10 dilution with PBS containing 5 mg/ml BSA and 10 mM sodium azide) and a fluorescence-activated flow cytometer (Profile; Coulter Corp., Epics Division, Hialeah, FL) (after a 1:1 dilution in the same buffer). The percentage of Covaspheres found in mixed aggregates was calculated from the relative fluorescence intensity of the aggregates in both colors (for details see Kuhn et al., 1991).

### Cell Cultures

DRGs were dissected from 10-d-old chicken embryos and dissociated after enzymatic digestion (Sonderegger et al., 1985). For immunocytochemical staining, *in situ* hybridization, and binding of protein-conjugated Covaspheres, the cells were plated on 35-mm cell culture dishes (Nunc, Roskilde, Denmark) that had been coated with 500  $\mu$ g/ml collagen S (type I) from calf skin (Boehringer Mannheim GmbH, Mannheim, Germany). To limit the volume of the medium to 250  $\mu$ l, donut-shaped Teflon insets were placed into the culture dishes as previously described (Kuhn et al., 1991). 20,000 cells per well were plated in MEM (GIBCO BRL) containing 5% heat-inactivated horse serum (GIBCO BRL), 5% chicken embryo extract, 20 ng/ml NGF, 10  $\mu$ g/ml BSA, 100  $\mu$ g/ml transferrin, 10  $\mu$ g/ml insulin, 20 ng/ml triiodothyronine, 40 nM progesterone, 0.2  $\mu$ g/ml corticosterone, 200  $\mu$ M putrescine, and 60 nM sodium selenite. After a cultura-

tion time of 20 h, the DRG cells were processed for immunocytochemical staining, in situ hybridization or binding of protein-conjugated Covaspheres.

Pure DRG nonneuronal cultures were prepared using a shaking method (McCarthy and Partlow, 1976). Ganglia were collected in PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , containing 0.5 g/liter glucose. After enzymatic digestion and dissociation (Sonderegger et al., 1985), the cells were plated at a density of 40,000 cells/Teflon inset on collagen-coated dishes. Immediately after plating, the culture dishes were placed on a horizontal laboratory shaker (Mini-Shaker; Adolf Kühner AG, Birsfelden, Switzerland) in the cell culture incubator ( $37^{\circ}\text{C}$ , 10%  $\text{CO}_2$ ), and agitated at 100 rpm for 30 s every 10 min for 1 h. Neuronal cells, which did not attach to the substratum but formed aggregates in the supernatant, were removed. Fresh medium was added to the adherent nonneuronal cells, which were examined by phase-contrast microscopy to determine whether all neurons had been removed. Residual phase-bright neurons were removed by rinsing the dishes with medium.

Embryonic fibroblasts were dissected from 10-d-old chicken embryos and grown in MEM containing 10% heat-inactivated FCS (GIBCO BRL) on collagen-coated cell culture dishes. After 7 d in culture, fibroblasts were replated and, after 1 d, used for binding studies with Covaspheres and for immunocytochemical staining.

For analysis of the formation of contact sites between neurites and peripheral glial cells, dissociated DRG cells (15,000 cells per well) were cultivated on laminin substratum in a serum-free medium, as described by Stoeckli et al. (1991). Laminin (GIBCO BRL) was adsorbed to poly-D-lysine-coated cell culture dishes, prepared as previously outlined (Kuhn et al., 1991). For coating, circular areas of  $0.5\text{ cm}^2$  were covered with  $20\text{ }\mu\text{l}$  of laminin at a concentration of  $20\text{ }\mu\text{g/ml}$  in PBS, and incubated for 2 h at  $37^{\circ}\text{C}$  in a humidified cell culture incubator. After two washes with PBS, the plates were blocked by incubation with  $10\text{ mg/ml}$  ovalbumin (Sigma Chemical Co.) in PBS for 30 min. Immediately before plating the cells, the blocking solution was removed and the plates were washed with PBS. Fab fragments of goat anti-axonin-1 IgG, rabbit anti-Nr-CAM IgG, goat anti-Ng-CAM IgG, rabbit anti-F11 IgG (anti-F11 Fabs were kindly provided by Dr. F. G. Rathjen, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany), and goat preimmune IgG were added at a concentration of  $250\text{ }\mu\text{g/ml}$  at the time of plating. For analysis of neurite-glia interactions at a higher magnification, dissociated DRG cultures were examined by scanning EM. In these cases, cells were grown on poly-D-lysine/laminin-coated glass coverslips. The cells were fixed with  $0.25\%$  glutaraldehyde in  $0.15\text{ M}$  sodium cacodylate (Fluka, Buchs, Switzerland), pH 7.2, for 30 min at RT, followed by an incubation in  $2\%$  glutaraldehyde in  $0.05\text{ M}$  sodium cacodylate for 30 min at RT and overnight at  $37^{\circ}\text{C}$ . After two washing steps with  $0.15\text{ M}$  sodium cacodylate and fixation with  $1\%$   $\text{OsO}_4$  in  $0.05\text{ M}$  sodium cacodylate for 1 h at RT, the coverslips were processed for scanning EM by critical point drying and gold sputtering.

### Binding of Protein-conjugated Covaspheres to Cultured Cells

After cultivation for 20 h, dissociated DRG cells were washed once with fresh culture medium and once with serum-free medium (Stoeckli et al., 1991) without NGF. Protein-conjugated Covaspheres were diluted 1:1,000 in the same serum-free medium. Incubation with the Covaspheres was carried out as described elsewhere (Kuhn et al., 1991). After a 1-h incubation at  $37^{\circ}\text{C}$ , 10%  $\text{CO}_2$ , the unbound Covaspheres were removed by three washes with serum-free medium. The cells were fixed for 1 h at  $37^{\circ}\text{C}$  with  $2\%$  paraformaldehyde,  $0.1\%$  glutaraldehyde in  $12.5\text{ mM}$  sodium cacodylate, pH 7.2, by adding a fourfold stock solution to the medium. After two washing steps with PBS, DRG cells were mounted in  $0.1\text{ M}$  Tris, pH 9.0,  $70\%$  glycerol,  $0.24\text{ M}$  *n*-propyl-gallate (Sigma Chemical Co.), cover-slipped, and inspected using a microscope (Leitz DMRXE; Leica, Heerbrugg, Switzerland) equipped with fluorescence optics. For scanning EM inspections, DRG cells were cultivated on poly-D-lysine/laminin-coated glass coverslips, incubated with protein-conjugated Covaspheres, fixed and processed for scanning EM as described above.

Preincubation of the cells with Fab fragments of polyclonal IgG was carried out at a concentration of  $500\text{ }\mu\text{g/ml}$  Fab in serum-free medium for 2 h at  $37^{\circ}\text{C}$ , 10%  $\text{CO}_2$ . Preincubation with rabbit anti-F11 IgG 4024 (kindly provided by Dr. F. G. Rathjen) and IgG of monoclonal anti- $\beta_1$ -integrin antibody JG22 (hybridomas producing JG22 were purchased from the Developmental Studies Hybridoma Bank, John Hopkins University School of Medicine, Baltimore, MD) was performed at the same concentration as with the Fab fragments. Polyclonal rabbit antisera against

tenascin (kindly provided by Dr. M. Chiquet, Biocenter, Basel, Switzerland), fibronectin, and laminin were added at a dilution of 1:20 in serum-free medium. After removal of antibody solutions, the cells were washed twice with serum-free medium before incubation with Covaspheres. Covaspheres were preincubated with Fab fragments as described for the Covasphere aggregation assay.

For quantification of axonin-1 Covaspheres binding to neurites, the number of beads on neurites was analyzed over a total neurite length of 10 mm using a Leitz DMRXE fluorescence microscope equipped with a CoolView camera (Photonic Science Ltd., Robertsbridge, UK). Neurite lengths were measured with the Image 1.49 (NIH) software on an Apple Macintosh Quadra 950 computer. The number of axonin-1 Covaspheres per millimeter neurite was compared among the different experimental conditions. For quantification of Covaspheres binding to nonneuronal cells, at least 100 cells were analyzed. A nonneuronal cell was determined as binding, if at least five axonin-1 Covaspheres were found on the surface of the cell. In control cultures without antibody preincubation, 70% of the nonneuronal cells bound axonin-1 Covaspheres.

### Immunocytochemical Stainings

For immunocytochemical localization of axonin-1, Nr-CAM, Ng-CAM, HNK-1, and  $\text{P}_0$ , DRG cells were fixed as described above after their incubation with protein-conjugated Covaspheres. After removal of the fixing solution, cells were washed three times with PBS and blocked by incubation with PBS containing 10% FCS for 30 min at RT. All antibodies were diluted in PBS containing 10% FCS. Primary antibodies were incubated for 3 h at RT. Monoclonal HNK-1 supernatant (hybridomas producing HNK-1 were obtained from the American Type Culture Collection, Rockville, MD) was added undiluted. The monoclonal antibody 1E8 (ascites fluid) against  $\text{P}_0$  was used at a dilution of 1:50 (1E8 was kindly provided by Dr. N. Ratner, University of Cincinnati College of Medicine, Cincinnati, OH). Goat anti-axonin-1 and goat anti-Ng-CAM antisera were incubated at a dilution of 1:1,000, rabbit anti-Nr-CAM antiserum at 1:500. After three washes with PBS, secondary antibodies were incubated for 2 h at RT. mAbs were detected by FITC-conjugated goat anti-mouse IgG (Cappel, Organon Teknica, Turnhout, Belgium) used at 1:500 or by Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) used at 1:250. Goat antibodies were detected by consecutive incubations with rabbit anti-goat IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) at 1:250 and FITC-conjugated goat anti-rabbit IgG (Cappel) at 1:500. Rabbit antibodies were detected by FITC-conjugated goat anti-rabbit IgG (Cappel) used at 1:500. After three washes with PBS, cells were processed for examination with the fluorescence microscope as described for binding of Covaspheres to cells. For double labeling with Covaspheres and antibodies, the cells were first incubated with Covaspheres, and after fixing, were immunocytochemically stained as described above. To determine the percentage of stained cells (e.g., axonin-1 binding HNK-1-positive cells) 100 cells per experiment were analyzed. No staining was observed on the cells upon incubation with preimmune sera or without primary antibodies.

### In Situ Hybridization

In situ hybridization was carried out with axonin-1 and Nr-CAM antisense complementary RNA (cRNA) probes labeled with digoxigenin (DIG)-11-UTP (Boehringer Mannheim GmbH) following the protocol of Schaeren-Wiemers and Gerfin-Moser (1993). The axonin-1 full-length cDNA clone d-509 (Zuellig et al., 1992; Giger et al., 1995) and a Nr-CAM cDNA clone spanning the six Ig domains were used as templates for in vitro transcription. The Nr-CAM cDNA clone was obtained by PCR amplification of random hexamer-primed cDNA derived from E14 chicken retina mRNA using the following primers: forward primer AB6F (5'-AGTAGAGCTCGCCACCATGATGAAAGAGAAGAGC-3') and backward primer AB21B (5'-CTTCTCCTGGTACCCATGAGAGTTC-3'). This PCR fragment was subcloned into Bluescript vector (pBluescript KS-, Stratagene Corp., La Jolla, CA), and sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH). In vitro transcription was performed at  $37^{\circ}\text{C}$  for 2 h in a reaction vol of  $50\text{ }\mu\text{l}$ . The reaction mix contained  $30\text{ }\mu\text{l}$   $\text{H}_2\text{O}$ -DEPC,  $5\text{ }\mu\text{l}$  linearized DNA ( $1\text{ }\mu\text{g}$ ),  $5\text{ }\mu\text{l}$   $10\times$  transcription buffer ( $400\text{ mM}$  Tris, pH 8.0,  $60\text{ mM}$   $\text{MgCl}_2$ ,  $100\text{ mM}$  dithiothreitol,  $20\text{ mM}$  spermidine),  $5\text{ }\mu\text{l}$   $10\times$  NTP-DIG mix (Boehringer Mannheim GmbH),  $1\text{ }\mu\text{l}$  RNasin ( $40\text{ U}/\mu\text{l}$ ; Promega, Catalys AG, Wallisellen, Switzerland), and  $4\text{ }\mu\text{l}$  RNA polymerase (T7 or T3, Boehringer Mannheim). Corresponding sense cRNA probes were prepared as controls.

DRG cells were washed with fresh medium and PBS, and fixed with 4% paraformaldehyde in PBS for 15 min at 37°C. Hybridization was performed overnight at 68°C with 40 ng/ml DIG-labeled axonin-1 cRNA or 1 µg/ml DIG-labeled Nr-CAM cRNA. The washing procedure was modified as follows from the original protocol: culture dishes were washed with 5× SSC for 10 min at RT, with 2× SSC for 20 min at 42°C, with 2× SSC for 20 min at 68°C, with 0.2× SSC, 15% formamide for 45 min at 68°C, and finally with 0.2× SSC for 5 min at RT. DIG-labeled cRNA hybrids were immunologically detected by incubation with sheep anti-DIG Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim GmbH) at a dilution of 1:5,000 in blocking buffer (0.1 M Tris, pH 7.5, 0.15 M NaCl, 2% FCS, 0.3% Triton X-100) for 1 h at RT. After color reaction overnight, cells were inspected on an inverted microscope (Nikon, Kusnacht, Switzerland) using both Hoffman modulation and bright-field optics.

### Analysis of Neurite–Glia Interaction

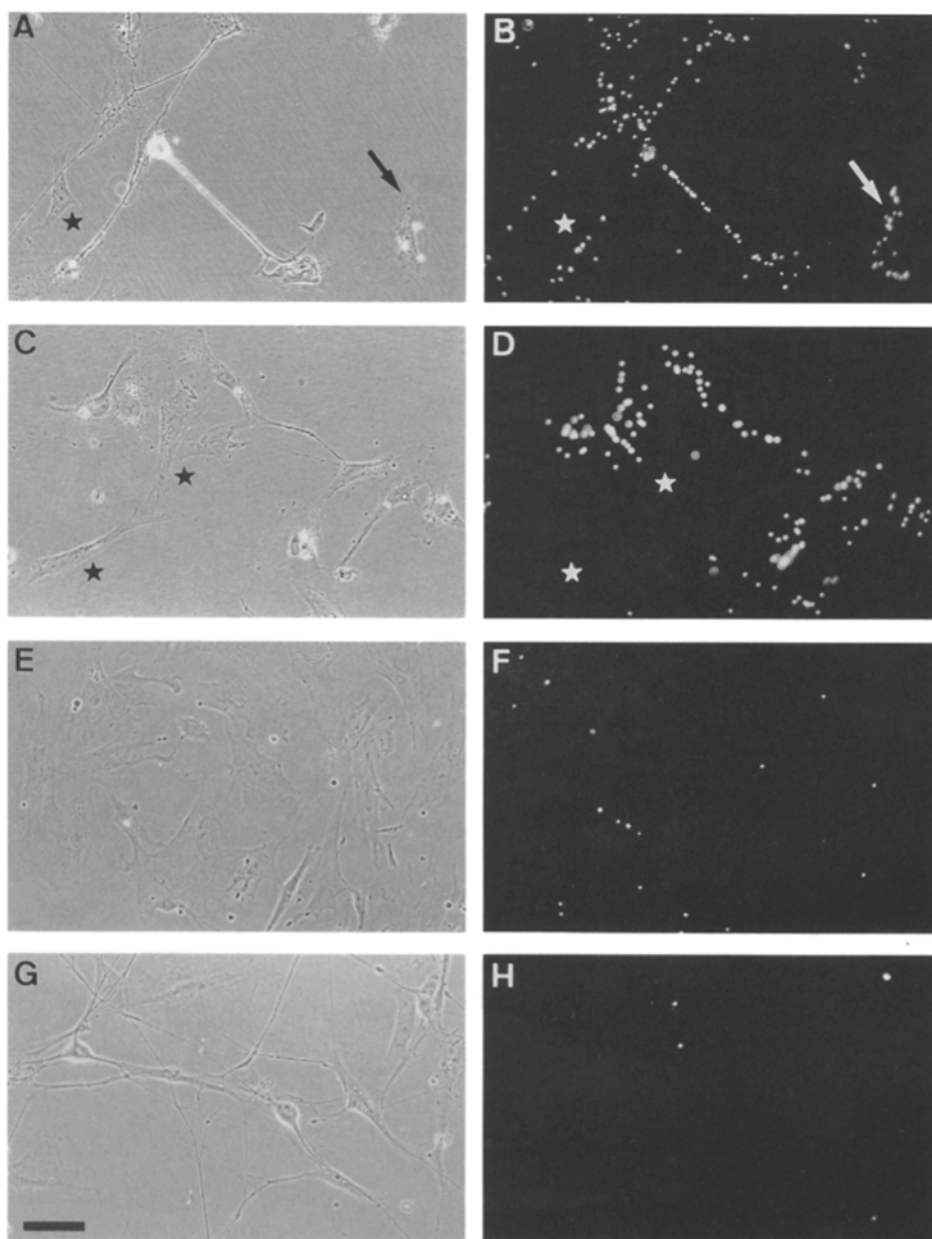
DRG cells grown for 20 h on laminin in serum-free medium in the presence of different Fab fragments were fixed with 2% glutaraldehyde for 1 h at 37°C, washed three times with PBS, and subsequently examined on an inverted microscope (Nikon) using phase-contrast optics. For each experi-

mental condition, two different culture dishes with similar cell densities were analyzed over a total area of 0.25 cm<sup>2</sup> with respect to the total numbers of neuronal and nonneuronal cells, and the number of glial cells in close contact with neurites. Neurons were identified by their phase-bright cell bodies, nonneuronal cells by their flat morphology. Contact sites were identified by the characteristic phase-bright halos that occurred in the phase-contrast optics when glial cells had wrapped around neurites. The ratios of the number of contact sites to the numbers of neuronal and nonneuronal cells were calculated for three different experiments and mean values and SEM were given.

## Results

### Peripheral Glial Cells from Dorsal Root Ganglia Bind Axonin-1

To identify cell-binding sites for axonin-1, dissociated DRG cells from 10-d-old chicken embryos were cultured on collagen substratum and incubated with fluorescent



**Figure 1.** Binding of axonin-1-coated Covaspheres to neurites and nonneuronal cells from dorsal root ganglia. Cultures of dissociated DRG cells and of fibroblasts were incubated with TRITC-labeled Covaspheres coated with purified axonin-1 and spheres binding was investigated by phase-contrast (*left*) and fluorescence optics (*right*). (*A* and *B*) Axonin-1-conjugated Covaspheres bound to neurites and to a subpopulation of nonneuronal cells (*arrow*). Nonneuronal cells with fibroblast-like morphology did not bind axonin-1-coated Covaspheres (*asterisk*). (*C* and *D*) Binding of axonin-1 Covaspheres to DRG nonneuronal cells was also observed in pure nonneuronal cultures. Nonbinding cells are marked with asterisks. (*E* and *F*) No binding of axonin-1 Covaspheres was detected on the surface of chicken embryonic fibroblasts. (*G* and *H*) Preincubation of axonin-1 Covaspheres with polyclonal goat anti-axonin-1 Fab fragments prevented the binding of axonin-1 beads to neurites and nonneuronal cells. Bar, 50 µm.

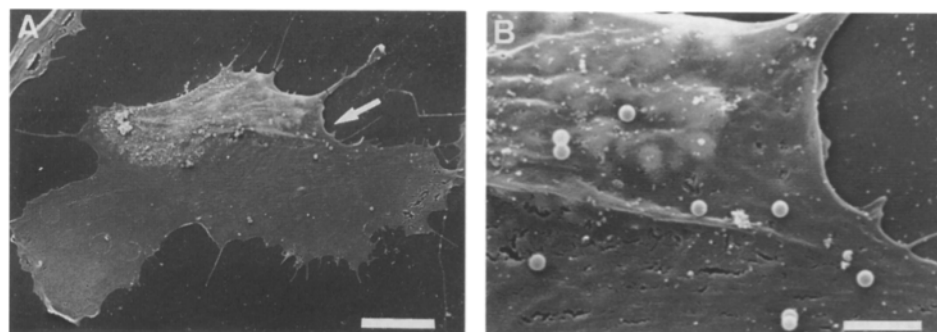
polystyrene microspheres (Covaspheres) carrying purified axonin-1 covalently coupled to their surface (Fig. 1). In these cultures, composed of  $\sim 1/3$  neuronal and  $2/3$  non-neuronal cells, binding of beads to neurites and neurite fascicles, as well as to a subpopulation of nonneuronal cells, was observed (Fig. 1, *A* and *B*). BSA-conjugated Covaspheres did not bind (data not shown). The axonin-1-binding nonneuronal cells represented 70% of all nonneuronal cells after 1 d in culture. Binding and nonbinding nonneuronal cells could be distinguished by their morphology; and there was a marked difference between them with respect to their association with neurites. The binding cells are polygonal, many of them bear processes, and they appear to represent a good substratum for outgrowing neurites; the nonbinding cells have a fibroblast-like morphology and are contacted by neurites much less frequently. In DRG cultures composed of nonneuronal cells only (see Materials and Methods), the percentage of cells binding axonin-1 Covaspheres remained unchanged (Fig. 1, *C* and *D*), providing evidence that the interaction between axonin-1 beads and nonneuronal cells is independent of neurites. The cell-type specificity of the axonin-1 Covasphere binding to nonneuronal cells was investigated by comparing different adherent cell types with flat morphology. Neither chicken embryonic fibroblasts (Fig. 1, *E* and *F*) nor CV-1 cells (data not shown) bound axonin-1-conjugated Covaspheres. Preincubation of axonin-1 beads with Fab fragments prepared from IgG of anti-axonin-1 antiserum prevented binding to neurites and nonneuronal cells of dissociated DRG cultures (Fig. 1, *G* and *H*). Scanning EM inspection of DRG cells incubated with axonin-1 beads revealed that Covaspheres on nonneuronal cells were in direct contact with the nonneuronal cell surface and not with thin neurites growing over the cells (Fig. 2). Hence, the binding of axonin-1 Covaspheres to nonneuronal cells exhibited the characteristics of a specific interaction between axonin-1 and a cell-surface receptor.

The axonin-1 binding nonneuronal cells were characterized by immunofluorescence. Nonneuronal cells of dissociated DRG cultures consist mainly of three distinct cell types: satellite cells, which are in vivo in close contact with the neuronal cell bodies of the ganglion; Schwann cells, which wrap around the axons and form their myelin

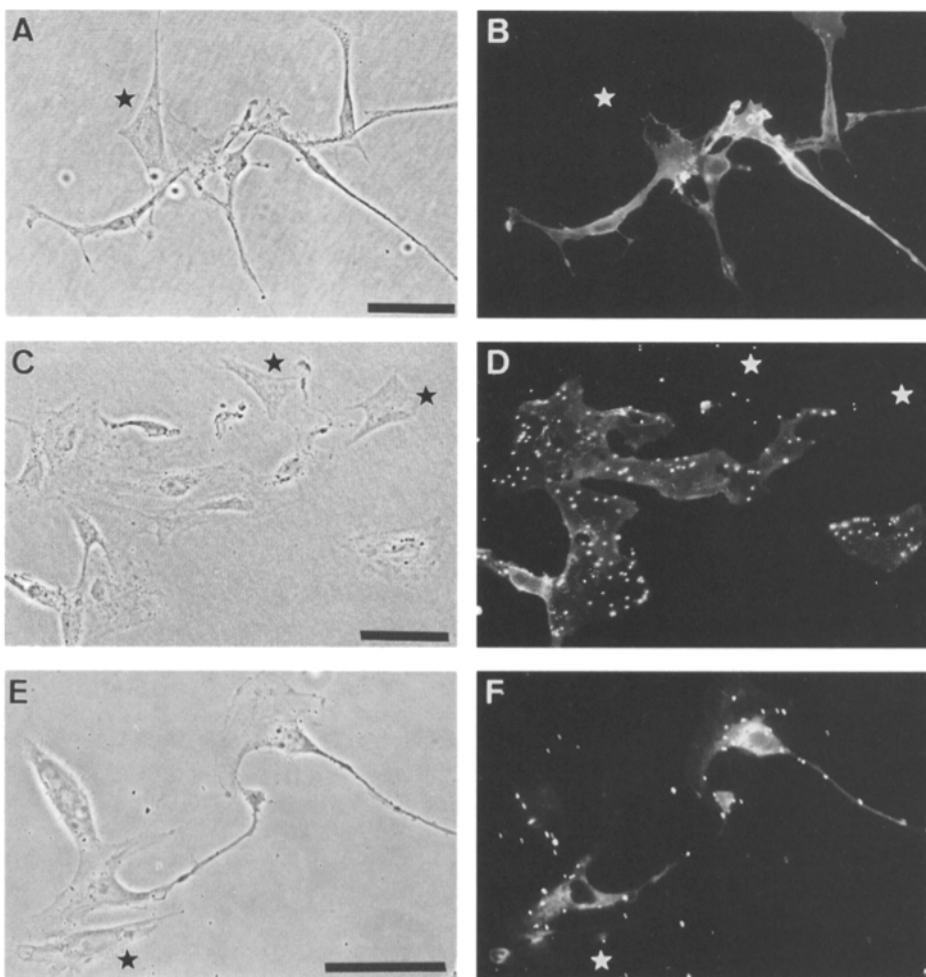
sheath; and fibroblasts, which are derived mainly from the connective tissue surrounding the ganglion. By immunolabeling with the mAb HNK-1, which is a marker for neural crest-derived cells including peripheral glial cells (Schwann and satellite cells) (Vincent et al., 1983),  $67 \pm 3\%$  (mean  $\pm$  SD,  $n = 4$ ) of the nonneuronal cells were stained (Fig. 3, *A* and *B*; for quantification see Materials and Methods). Combining HNK-1 immunolabeling and Covasphere binding, a good correlation between the presence of the HNK-1 epitope and the expression of the axonin-1 receptor was found (Fig. 3, *C* and *D*). Most ( $91 \pm 2\%$ ; mean  $\pm$  SD,  $n = 4$ ) of the HNK-1-positive nonneuronal cells bound axonin-1 Covaspheres at their surface, whereas most of the HNK-1-negative nonneuronal cells with fibroblast-like morphology did not. A substantial fraction of the axonin-1-binding nonneuronal cells exhibited positive immunocytochemical staining with a monoclonal antibody (1E8) against the myelin protein  $P_0$  (Fig. 3, *E* and *F*), which is an early Schwann cell marker that is transiently expressed in culture (Bhattacharyya et al., 1991, 1993). About 40% of all nonneuronal cells were  $P_0$  positive. Cultured chicken embryonic fibroblasts were found to be HNK-1 and  $P_0$  negative (data not shown) and did not bind axonin-1 Covaspheres (Fig. 1, *E* and *F*). These results identified the axonin-1-binding nonneuronal cells of the DRG cultures as Schwann and satellite cells, and in the following we designate them as peripheral glial cells.

#### ***Axonin-1 Receptor on Glial Cells Is Distinct from Axonin-1 and Ng-CAM***

Axonin-1 binds to Ng-CAM (Kuhn et al., 1991) and to other axonin-1 molecules (Rader et al., 1993). To identify the glial receptor of axonin-1, we tested whether Ng-CAM and axonin-1 are expressed by DRG glial cells. In situ hybridization of dissociated DRG cells with a digoxigenin-labeled axonin-1 antisense cRNA probe, revealed axonin-1 transcripts only in neuronal cells; no signals were detected in nonneuronal cells (Fig. 4 *A*). With the corresponding axonin-1 sense cRNA probe, staining was observed in neither neuronal nor nonneuronal cells (Fig. 4 *B*). The absence of axonin-1 mRNA in nonneuronal cells was in accordance with the absence of surface-exposed axonin-1 pro-



**Figure 2.** Evidence for a direct binding of axonin-1 Covaspheres to DRG nonneuronal cells. Dissociated DRG cells were cultivated on poly-D-lysine/laminin-coated glass coverslips for 20 h and incubated with axonin-1-conjugated Covaspheres. After fixation the cells were processed for scanning EM inspection. (*A*) The axonin-1 Covaspheres on nonneuronal cells were located directly on the cell surface and not on neurites growing over the cells. One part of the nonneuronal cell in (*A*) (arrow) is shown in (*B*) at a higher magnification. Bars, 10  $\mu$ m (in *A*); 2.5  $\mu$ m (in *B*).



**Figure 3.** Identification of DRG nonneuronal cells with axonin-1-binding sites as peripheral glial cells. Peripheral glial cells were identified by immunocytochemical staining using the mAb HNK-1, which labels neural crest-derived glial cells, and the mAb 1E8 against myelin protein P<sub>0</sub>. 1E8 is a marker for early chicken Schwann cells. Cellular binding sites for axonin-1 were localized by binding of axonin-1-coated Covaspheres. (A and B) Staining with the mAb HNK-1 and a secondary FITC-labeled antibody revealed that 67% of the nonneuronal cells were HNK-1 positive. A HNK-1-negative cell with fibroblast-like morphology is marked by an asterisk. (C and D) Double staining for HNK-1 (FITC) and axonin-1-binding sites (axonin-1 TRITC-Covaspheres; double exposure with both filter settings). Most (91%) of the HNK-1-positive nonneuronal cells bound axonin-1 Covaspheres, whereas most of HNK-1-negative cells did not (asterisks). (E and F) Double labeling for P<sub>0</sub> (detected with 1E8 and a secondary Cy3-labeled antibody) and axonin-1-binding sites (axonin-1 FITC-Covaspheres, double exposure). About 40% of all nonneuronal cells were P<sub>0</sub> positive. All the P<sub>0</sub>-positive cells bound axonin-1 Covaspheres. A P<sub>0</sub>-negative cell that did not bind axonin-1 beads is indicated by an asterisk. Bars, 50  $\mu$ m.

tein. Surface immunostaining with anti-axonin-1 antiserum was found on neurons but not glial cells (Fig. 4, C and D). If the cells were preincubated with anti-axonin-1 Fab fragments, binding of axonin-1 Covaspheres to neurites or to glial cells was not reduced (Fig. 4, I and K). This confirms earlier results that axonin-1 on neurites is not the receptor for axonin-1 Covaspheres (Kuhn et al., 1991) and excluded axonin-1 as a glial receptor for axonin-1. Immunostaining of DRG cells with anti-Ng-CAM antiserum revealed a weak expression of Ng-CAM by some glial cells (~10% of all nonneuronal cells), whereas neuronal membranes contained high concentrations of Ng-CAM (Fig. 4, E and F). To test if this small amount of glial Ng-CAM contributes to axonin-1 binding, dissociated DRG cells were preincubated with anti-Ng-CAM Fab fragments before a Covasphere cell-binding assay was performed. A strong inhibition of binding to the neurites (80–90%) but not to the glial cells was found (Fig. 4, G and H). In summary, the results suggested a molecule distinct from axonin-1 and Ng-CAM as the glial receptor for axonin-1.

#### **Antibody Perturbation Studies Identify Nr-CAM as a Receptor for Axonin-1 on both Glial Cells and Neurites**

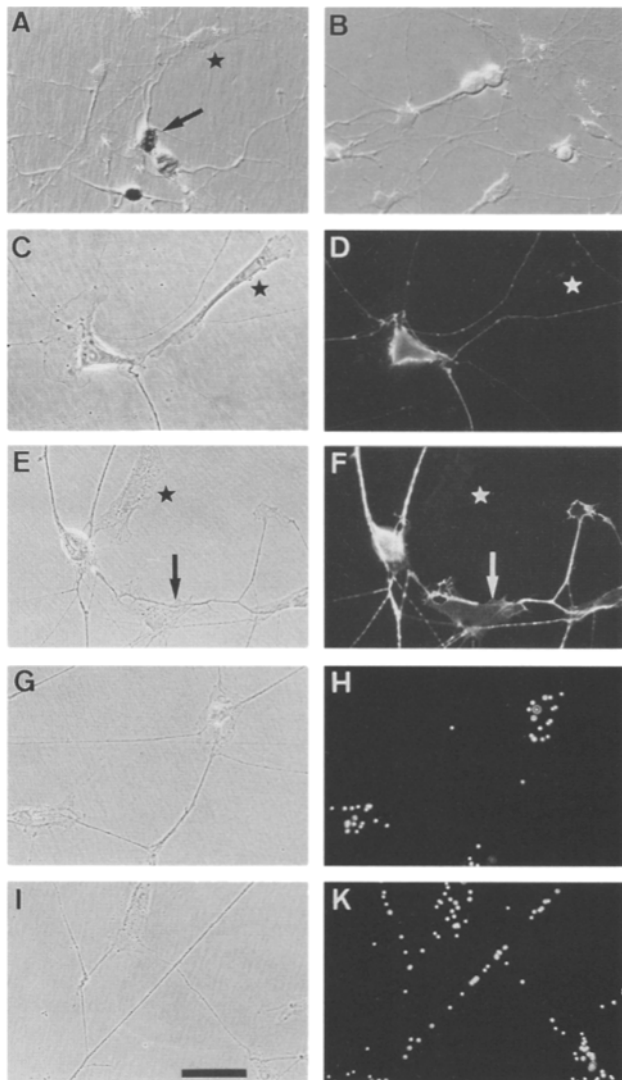
In our search for a glial axonin-1 receptor, we tested the

effect of several antibodies against ECM components and CAMs in the axonin-1 Covasphere cell-binding assay. Preincubation of the DRG cells with antisera against the ECM glycoproteins laminin, fibronectin, or tenascin did not interfere with the binding of axonin-1 Covaspheres (data not shown). Similarly, preincubation with IgG of the mAb JG22 directed against  $\beta_1$  integrin or of polyclonal antiserum against the Ig/FNIII glycoprotein F11 had no effect (data not shown). In contrast, preincubation of the cultures with anti-Nr-CAM antiserum or with corresponding Fab fragments (Fig. 5, C and D) resulted in a reduction of axonin-1 Covasphere binding to glial cells by ~70% (for quantification, see Materials and Methods), as compared with a control without antibody preincubation (Fig. 5, A and B) or with incubation of preimmune Fab fragments (Fig. 5, G and H). In addition, preincubation with anti-Nr-CAM Fab fragments resulted in a reduction of axonin-1 Covasphere binding to neurites by 20% (Fig. 5, E and F). In summary, these antibody perturbation experiments identified Nr-CAM as a receptor for axonin-1 on both glial cells and neurites.

#### **DRG Glial Cells and Neurons Express Nr-CAM**

The expression of Nr-CAM by DRG glial cells and neu-





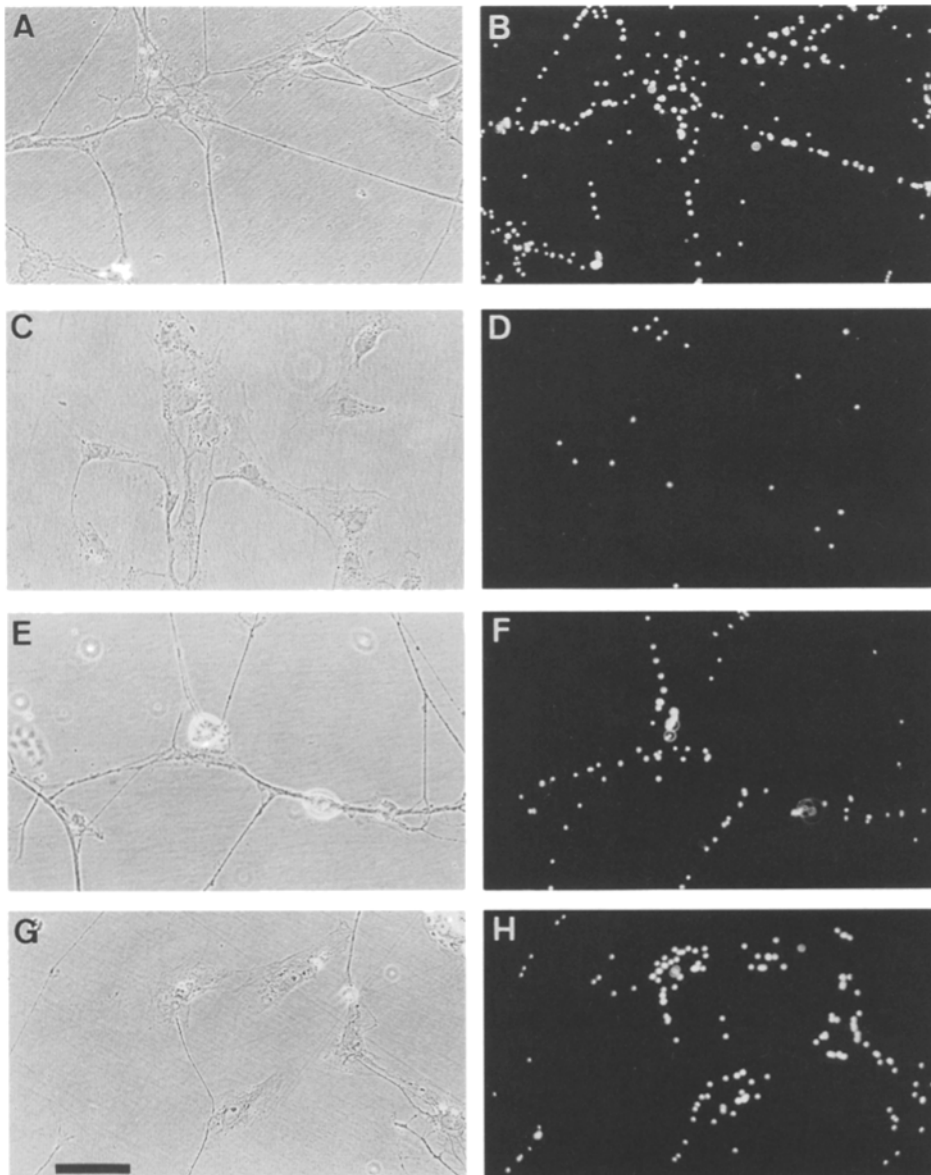
**Figure 4.** Characterization of the peripheral glial cell receptor for axonin-1 as a molecule distinct from axonin-1 and Ng-CAM. (A–D) Axonin-1 expression by cultured DRG neurons, but not glial cells, was detected by in situ hybridization (A and B; Hoffman modulation optics) and immunofluorescence staining (C and D). (A) In situ hybridization of DRG cells with an axonin-1 antisense probe revealed axonin-1 mRNA in neuronal cell bodies (arrow) but not in glial cells (asterisk). (B) No hybridization signal was observed with the axonin-1 sense probe. (C and D) Axonin-1 immunoreactivity after staining with goat anti-axonin-1 antiserum and a secondary FITC-labeled antibody was detected on neuronal cell bodies and neurites but not on glial cells (asterisk); (C) phase-contrast optics, (D) fluorescence optics of the same field. (E and F) DRG neurons and a subpopulation of DRG glial cells express Ng-CAM. Incubation with a goat anti-Ng-CAM antiserum and a secondary FITC-labeled antibody revealed strong Ng-CAM staining on neuronal cell bodies and neurites. Weak Ng-CAM immunoreactivity was found on a subpopulation of glial cells (arrow), whereas most glial cells were not stained (asterisk); (E) phase-contrast optics, (F) fluorescence optics of the same field. (G–K) The peripheral glial cell receptor for axonin-1 is different from Ng-CAM and axonin-1. (G and I) Phase-contrast micrographs of dissociated DRG cells; (H and K) fluorescent micrographs of the respective fields. (G and H) Preincubation of the cells with anti-Ng-CAM Fab fragments prevented the binding of axonin-1 TRITC-Covaspheres to neurites, but not to glial cells. (I and K) Preincubation of dissociated DRG cells with anti-axonin-1 Fab fragments did not prevent binding of axonin-1 Covaspheres to neurites or to glial cells. Bar, 50  $\mu$ m.

rons was investigated by immunocytochemistry and in situ hybridization. Immunofluorescence staining with anti-Nr-CAM antiserum revealed Nr-CAM on the surface of neurites and on the majority of the nonneuronal cells (Fig. 6, A and B). Nonneuronal cells expressed Nr-CAM to various levels. A correlation between Nr-CAM expression and axonin-1 Covasphere binding to glial cells was found (Fig. 6, C and D). In situ hybridization experiments confirmed our immunocytochemical data. For the detection of Nr-CAM mRNA in cultured DRG cells, a digoxigenin-labeled Nr-CAM antisense cRNA probe, transcribed from a cDNA fragment encoding the six Ig-like domains of Nr-CAM, was hybridized to the cells, revealing Nr-CAM transcripts in both neuronal and glial cells (Fig. 6, E and F). Glial cells in close contact with neurites were the most intensively stained of the nonneuronal cells, whereas fibroblast-like cells were negative. No signals were observed using the Nr-CAM sense probe (Fig. 6, G and H). Double immunostaining experiments revealed that the majority (~75%) of the Nr-CAM expressing nonneuronal cells were HNK-1 positive (Fig. 7). Therefore, we conclude that most of the Nr-CAM-positive nonneuronal cells are peripheral glial cells.

#### **Microspheres Aggregation Studies Reveal a Direct Binding between Axonin-1 and Nr-CAM**

The inhibition of binding of axonin-1-coated Covaspheres to peripheral glial cells and DRG neurites by anti-Nr-CAM antibodies identified Nr-CAM as a receptor for axonin-1. To investigate whether axonin-1 and Nr-CAM bind each other directly, we carried out binding assays with purified axonin-1, Nr-CAM, Ng-CAM, BSA, and nonimmune IgG, covalently coupled to TRITC- or FITC-labeled Covaspheres. Aggregate formation was analyzed using a fluorescence microscope and a fluorescence-activated flow cytometer (Fig. 8). Upon incubation of axonin-1- and Nr-CAM-conjugated Covaspheres, 50–60% of the beads were found in mixed aggregates (Fig. 8 A). Examination of those aggregates by fluorescence microscopy revealed that they contained axonin-1 and Nr-CAM beads in a homogeneous distribution (not shown). Binding between axonin-1 Covaspheres and Nr-CAM Covaspheres was found to be independent of divalent cations such as  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  (data not shown).

Each other combination of the Ig/FNIII-like glycoproteins axonin-1, Nr-CAM, and Ng-CAM was also included in this study to serve as controls. All results were in agreement with the published data. Axonin-1-coated Covaspheres formed mixed aggregates with Ng-CAM Covaspheres (Fig. 8 B), but did not self-aggregate (Fig. 8 C; see Kuhn et al., 1991). The percentage of Covaspheres found in mixed axonin-1/Ng-CAM aggregates was 70–80%. Nr-CAM-conjugated Covaspheres showed self-aggregation (Fig. 8 D), an observation consistent with a report on a homophilic interaction of Nr-CAM (Mauro et al., 1992). Ng-CAM and Nr-CAM Covaspheres did not form mixed aggregates (Fig. 8 E; Morales et al., 1993). Ng-CAM Covaspheres also bound in a homophilic manner (Fig. 8 F; Grumet and Edelman, 1988; Kuhn et al., 1991). On the basis of the number of beads incorporated into the mixed aggregates and since the coupling efficiencies were similar for the different proteins (~50%), we suggest that the ho-



**Figure 5.** Perturbation of the binding of axonin-1-coated Covaspheres to peripheral glial cells and to neurites by anti-Nr-CAM antibodies. Dissociated DRG cells were incubated with TRITC-labeled axonin-1-conjugated Covaspheres and spheres binding was investigated by phase-contrast (*left*) and fluorescence optics (*right*). (*A* and *B*) Binding of axonin-1 Covaspheres to neurites and glial cells in the absence of antibody. (*C–F*) Preincubation of DRG cells with anti-Nr-CAM Fab fragments resulted in a reduction in axonin-1 Covasphere binding to glial cells by 70% (*C* and *D*) and to neurites by 20% (*E* and *F*) revealing Nr-CAM both as a glial and neuritic axonin-1 receptor. (*G* and *H*) Preincubation of the cells with preimmune Fab fragments had no effect on the binding of axonin-1 beads. Bar, 50  $\mu$ m.

mophilic Nr-CAM binding was stronger than the homophilic Nr-CAM binding (60–70% versus 20–30% of the beads in mixed aggregates).

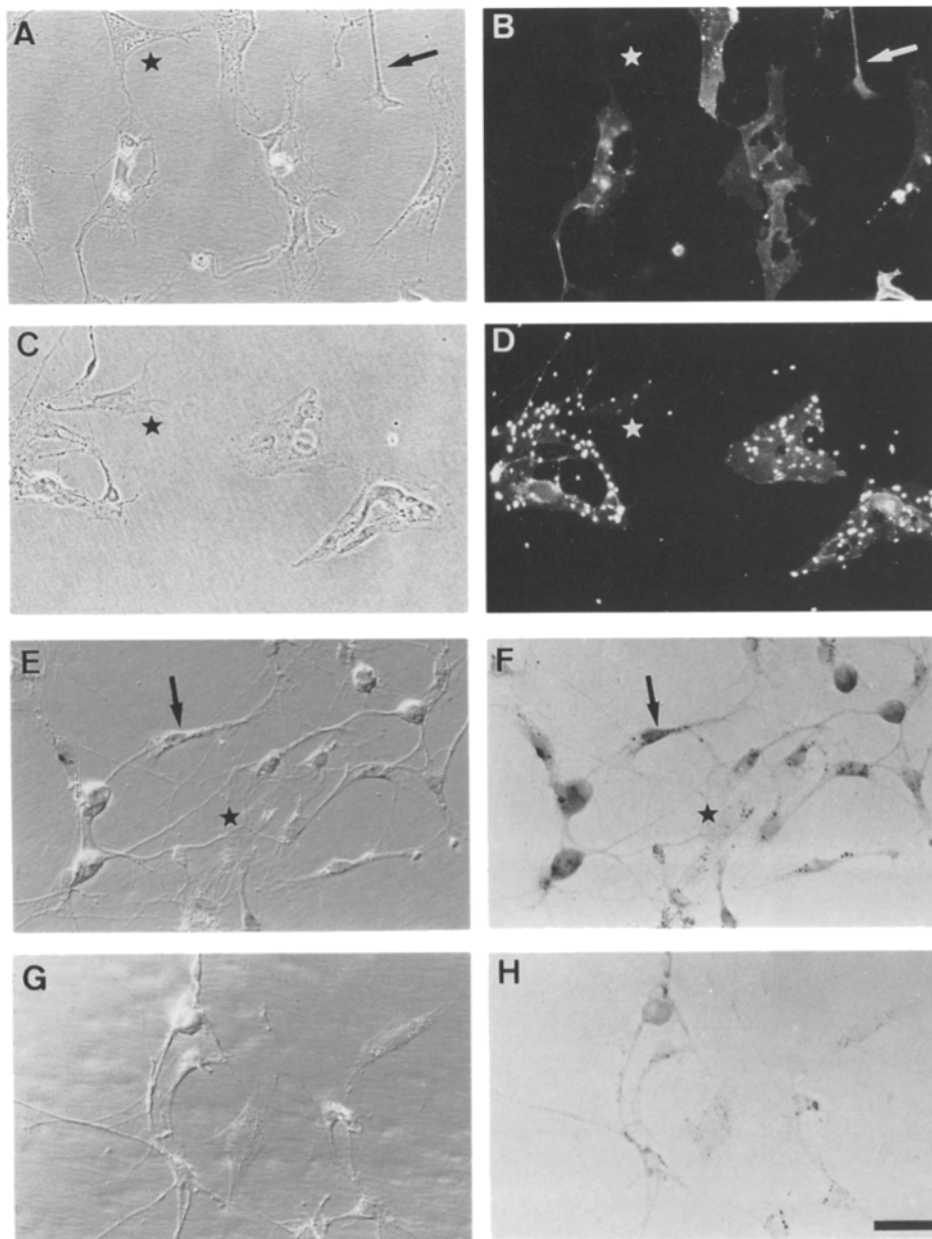
In addition to the above mentioned interactions of axonin-1, Nr-CAM, and Ng-CAM, several other controls provided evidence for the specificity of the binding between axonin-1 and Nr-CAM (Fig. 9). Neither axonin-1 nor Nr-CAM Covaspheres formed aggregates with Covaspheres coated with control proteins like BSA (Fig. 9, *A* and *F*) or nonimmune IgG (Fig. 9, *B* and *G*). Preincubation of axonin-1 Covaspheres with anti-axonin-1 Fab fragments or of Nr-CAM Covaspheres with anti-Nr-CAM Fab fragments prevented the formation of mixed aggregates (Fig. 9, *L* and *M*). Furthermore, no interference with the formation of mixed aggregates was observed if preimmune or anti-Nr-CAM Fab fragments were used (data not shown). Preincubation of axonin-1 Covaspheres with anti-Nr-CAM Fab fragments did not alter the formation of mixed aggregates with Nr-CAM Covaspheres (Fig. 9 *N*). Similarly, the

formation of mixed aggregates with axonin-1 Covaspheres was unaffected if Nr-CAM Covaspheres were preincubated with anti-axonin-1 Fab fragments (Fig. 9 *O*). Therefore, a contamination of the axonin-1 preparations with Nr-CAM or of the Nr-CAM preparations with Ng-CAM can be excluded as an explanation for the formation of mixed axonin-1/Nr-CAM aggregates. Taken together, the results demonstrate that axonin-1 and Nr-CAM can bind each other directly.

#### ***Axonin-1 and Nr-CAM Are Involved in the Formation of Contacts between Glial Cells and Neurites***

To investigate whether binding between neuritic axonin-1 and glial Nr-CAM has a functional relevance for the interaction between neurons and glial cells, we cultured dissociated DRG cells on laminin substratum in the presence of anti-axonin-1 or anti-Nr-CAM Fab fragments. In the absence of antibodies a number of glial cells were observed



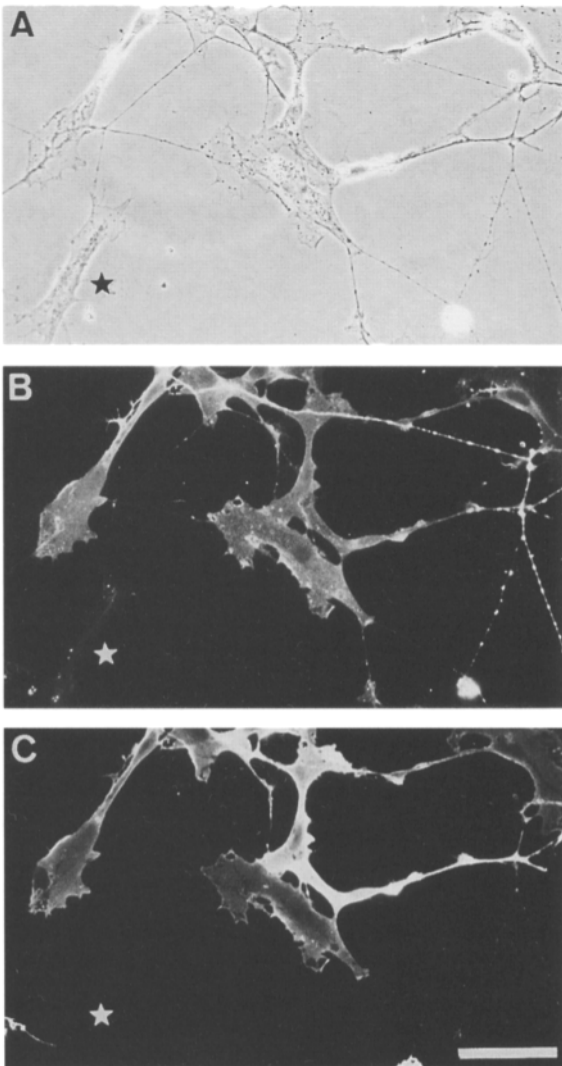


**Figure 6.** Expression of Nr-CAM by peripheral glial cells and neurons, and colocalization with axonin-1-binding sites. Nr-CAM expression of DRG glial cells and neurons was analyzed by immunofluorescence staining (*A–D*) and in situ hybridization (*E–H*). (*A* and *C*) Phase-contrast micrographs of dissociated DRG cells; (*B* and *D*) fluorescence micrographs of the same fields. (*B*) Detection of Nr-CAM immunoreactivity on neurites (arrow) and on glial cells after incubation with a rabbit anti-Nr-CAM antiserum and a secondary FITC-labeled antibody. Nonneuronal cells with fibroblast-like morphology were Nr-CAM negative (asterisk). (*D*) Nr-CAM expressing glial cells bind axonin-1 TRITC-Covaspheres (double exposure with both filter settings). A Nr-CAM-negative cell that did not bind axonin-1 Covaspheres over background level is marked by an asterisk. (*E* and *G*) Hoffman modulation optics; (*F* and *H*) bright field optics of the corresponding fields. (*E* and *F*) In situ hybridization with the Nr-CAM antisense probe revealed staining of neuronal cell bodies and of glial cells that are in close contact with neurites (arrow). Nonneuronal cells with fibroblast-like morphology were not stained (asterisk). (*G* and *H*) No staining was observed if the cells were hybridized with the Nr-CAM sense probe. Bar, 50  $\mu$ m.

in close contact with neurites after one day of culture. At lower magnification in phase-contrast optics, such sites, referred to as contact sites, were readily localized by their phase-bright halos (Fig. 10 *A*). Scanning EM inspection of such cultures confirmed the close association of these glial cells with neurites (Fig. 10 *B*) and revealed that many had started to ensheath the neurite. Antibody perturbation studies demonstrated that axonin-1 and Nr-CAM are involved in the formation of these contacts between DRG neurites and glial cells. In the presence of anti-axonin-1 (Fig. 10 *C*) or anti-Nr-CAM (Fig. 10 *D*) Fab fragments, the number of contact sites was markedly reduced, whereas preimmune, anti-Ng-CAM or anti-F11 Fab fragments did not affect the number of contact sites. None of the Fab preparations used caused a reduction in the lengths of the neurites. Since neurite outgrowth is very sensitive to a putative toxin in the medium, a general toxic

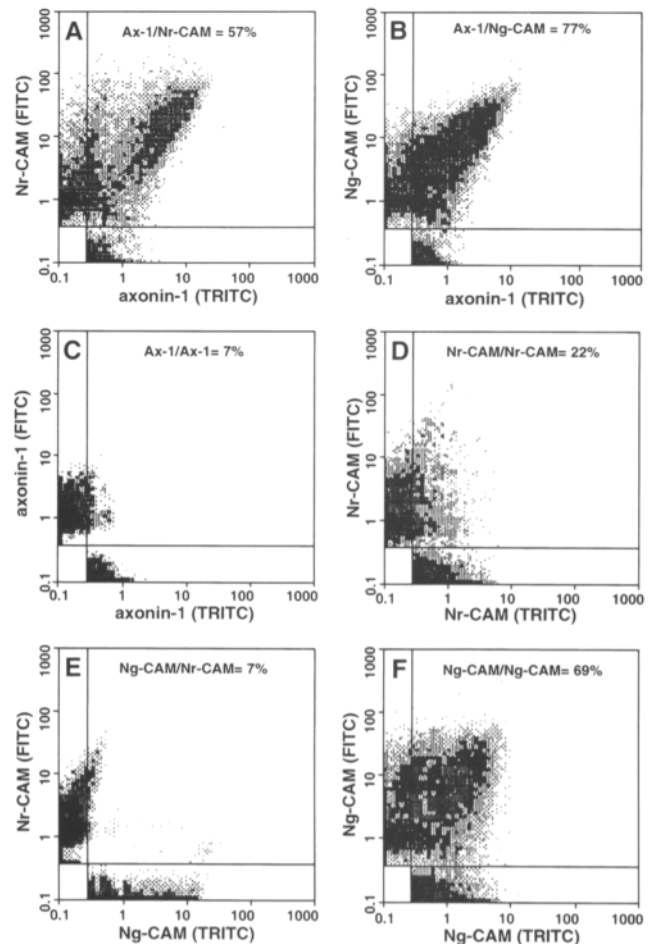
effect can be excluded as an explanation for the observed results.

A quantitative assessment of these observations is given in Fig. 11. For each experimental condition, the total numbers of neurons, of nonneuronal cells including satellite cells, Schwann cells, and fibroblasts, and the total number of contact sites (glial cells in close contact with neurites) were determined in defined plating areas with similar cell density (see Materials and Methods). In all experimental conditions  $\sim 500$  neurons were observed. The numbers of neurites per neuron and the neurite lengths were similar under all conditions, and the numbers of nonneuronal cells were between 850 and 1250. For normalization, we calculated the ratio of the number of contact sites to the total number of nonneuronal cells and to the total number of neurons. In control cultures approximately a quarter of all neurons exhibited at least one neurite/glia contact and ap-



**Figure 7.** Correlation between the expression of Nr-CAM and of the HNK-1 epitope by peripheral glial cells. Dissociated DRG cells were analyzed for the expression of Nr-CAM and of the HNK-1 epitope (as marker for peripheral glial cells) by double immunofluorescence. (A) phase-contrast optics; (B and C) fluorescence optics of the same field. (B) Labeling of nonneuronal cells and neurites with rabbit anti-Nr-CAM antiserum and a secondary FITC-labeled antibody. (C) Staining of peripheral glial cells with the mAb HNK-1 and a secondary Cy3-labeled antibody. Most of the Nr-CAM expressing cells were found to be HNK-1 positive. A double-negative nonneuronal cell is indicated by an asterisk. Bar, 50  $\mu$ m.

proximately a tenth of the nonneuronal cells were in contact with a neurite (Fig. 11). The presence of anti-axonin-1 and anti-Nr-CAM Fab fragments reduced the number of contact sites per neuron by 60 and 70%, respectively, and the number of contact sites per nonneuronal cell by 57 and 61%, respectively, when compared to the control without antibodies. Using anti-Ng-CAM, anti-F11 or preimmune Fab fragments, the ratios were identical to those observed with the controls. Similar results were obtained using corresponding IgGs instead of Fab fragments (not shown). In summary, the experiments implicate a role for axo-

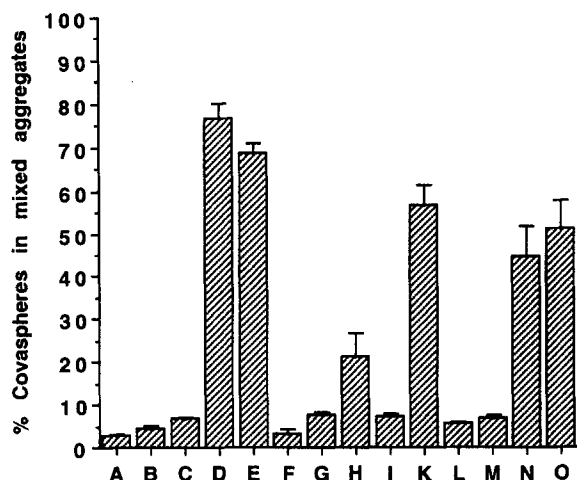


**Figure 8.** Formation of mixed aggregates between Covaspheres coated with purified axonin-1 and Nr-CAM. Covaspheres (TRITC or FITC) covalently coupled with axonin-1, Nr-CAM, or Ng-CAM were incubated in all possible dual combinations at a 1:1 ratio and the formation of aggregates was analyzed using a fluorescence-activated cell sorter. The state of aggregation is visualized by a two-dimensional dot blot representation of the relative fluorescence intensity given in a logarithmic scale (x-axis, red fluorescence; y-axis, green fluorescence). The numbers at the top of each histogram represent the calculated percentages of beads of both species found in mixed aggregates (mean values of at least four independent experiments). (A) Formation of mixed aggregates between axonin-1 and Nr-CAM Covaspheres. (B) Axonin-1 Covaspheres formed heterophilic aggregates with Ng-CAM Covaspheres. (C) Axonin-1 Covaspheres did not form homophilic aggregates. (D) Homophilic binding between Nr-CAM Covaspheres. (E) No formation of mixed aggregates was observed between Ng-CAM and Nr-CAM Covaspheres. (F) Homophilic aggregation of Ng-CAM Covaspheres.

nin-1 and Nr-CAM in the formation of close neurite/glia contacts.

## Discussion

We report on a binding between the Ig superfamily glycoproteins axonin-1 and Nr-CAM and present evidence that this binding mediates contact formation between neurites expressing axonin-1 and peripheral glial cells expressing



**Figure 9.** Characterization of the binding between purified axonin-1 and Nr-CAM as a specific molecular interaction. The aggregation of axonin-1- and Nr-CAM-conjugated Covaspheres with each other and with other proteins, such as Ng-CAM, BSA, and nonimmune IgG, covalently coupled to Covaspheres was assayed by coincubation, followed by the determination of the percentage of Covaspheres (of both species) found in mixed aggregates using a flow cytometer. Mean  $\pm$  SEM for at least four different experiments is given. If one species of Covaspheres was preincubated with Fab fragments, the unbound antibodies were removed before adding the other Covasphere species. Several controls indicate that binding between axonin-1 and Nr-CAM is a specific molecular interaction. (A) Axonin-1 versus BSA; (B) axonin-1 versus nonimmune IgG; (C) axonin-1 versus axonin-1; (D) axonin-1 versus Ng-CAM; (E) Ng-CAM versus Ng-CAM; (F) Nr-CAM versus BSA; (G) Nr-CAM versus nonimmune IgG; (H) Nr-CAM versus Nr-CAM; (I) Nr-CAM versus Ng-CAM; (K) axonin-1 versus Nr-CAM; (L) axonin-1 preincubated with anti-axonin-1 Fab fragments versus Nr-CAM; (M) Nr-CAM preincubated with anti-Nr-CAM Fab fragments versus axonin-1; (N) axonin-1 preincubated with anti-Nr-CAM Fab fragments versus Nr-CAM; (O) axonin-1 versus Nr-CAM preincubated with anti-axonin-1 Fab fragments.

Nr-CAM. These results implicate axonin-1 and Nr-CAM as mediators of neuron-glia interaction during the initial stage of axon ensheathment in the peripheral nervous system (PNS).

### **Axonin-1 and Nr-CAM Bind Each Other**

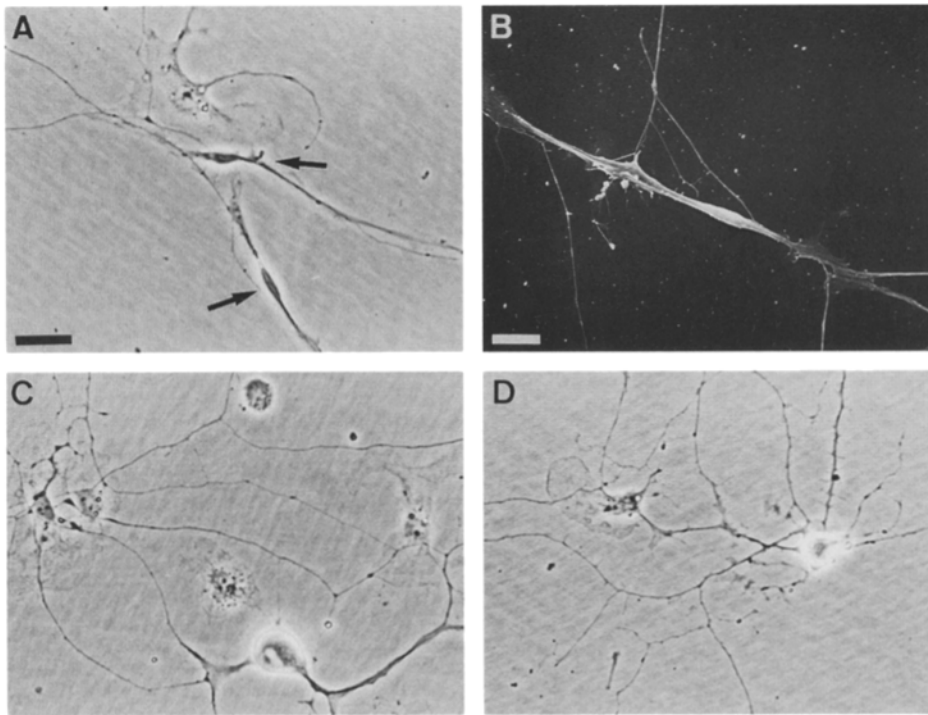
Initial cell-cell and cell-matrix contacts are generally mediated by relatively weak molecular interactions of CAMs or between cell-surface receptors and ECM components (Edelman and Crossin, 1991; Brümmendorf and Rathjen, 1993; van der Merwe and Barclay, 1994). Thus, assay systems based on multiple interactions are best suited to the identification of binding partners for CAMs. For the past number of years, protein-coated fluorescent beads (Covaspheres) have been used by several laboratories to detect interactions between neural CAMs (Grumet and Edelman, 1988; Kuhn et al., 1991; Mauro et al., 1992; Brümmendorf et al., 1993; Morales et al., 1993). Using Covaspheres with covalently coupled axonin-1 and cultures of dissociated chicken DRG cells, we monitored binding of axonin-1 not only to neurites but also to a subpopulation

of nonneuronal cells. Several lines of evidence identified the axonin-1-binding nonneuronal cells as peripheral glial cells including Schwann cells and satellite cells but excluding fibroblasts derived from the connective tissue surrounding the ganglion.

The glial receptor for axonin-1 was identified as Nr-CAM, another neural cell-surface glycoprotein of the Ig/FNIII-family. In the search for the glial axonin-1 receptor, we first tested Ng-CAM and axonin-1 itself, both previously identified axonin-1-binding partners (Kuhn et al., 1991; Rader et al., 1993). Axonin-1 could be excluded as the glial receptor as it was not detected on the surface of glial cells, while, consistent with earlier findings on Ng-CAM-expression by Schwann cells (Daniloff et al., 1986), we found weak Ng-CAM-immunoreactivity on a small subpopulation of DRG non-neuronal cells; antibody perturbation studies and quantitative considerations indicate, however, that Ng-CAM is not a major glial axonin-1 receptor.

During the past five years, an increasing number of heterophilic interactions have been reported among Ig/FNIII-like family members, including binding between L1 and N-CAM (Kadmon et al., 1990), axonin-1 and Ng-CAM (Kuhn et al., 1991), F11 and Ng-CAM (Brümmendorf et al., 1993), and between F11 and Nr-CAM (Morales et al., 1993). Likewise, reports on interactions between Ig/FNIII-like glycoproteins and ECM components have become numerous, including binding between N-CAM and heparin (Cole and Akeson, 1989), F11 and restrictin (Rathjen et al., 1991; Brümmendorf et al., 1993), F11 and tenascin (Zisch et al., 1992), Ng-CAM and laminin (Grumet et al., 1993), and Ng-CAM and neurocan, as well as between N-CAM and neurocan (Friedlander et al., 1994). In addition, it has recently been shown by antibody perturbation studies that neurite outgrowth on TAG-1 as a substratum involves an L1-like molecule and  $\beta_1$ -integrin (Felsenfeld et al., 1994). These findings prompted us to investigate whether different CAMs (Nr-CAM, F11), ECM proteins (tenascin, fibronectin, laminin), and  $\beta_1$ -integrin could function as a glial receptor for axonin-1. Cultured DRG cells were incubated with antibodies against these molecules before the axonin-1 Covasphere cell-binding assay. Only anti-Nr-CAM antibodies inhibited the binding of axonin-1-coated Covaspheres to glial cells by  $\sim 70\%$ . Thus far, it is unclear whether the residual binding in the case of the anti-Nr-CAM antibodies is due to an additional axonin-1 ligand. In view of the increasing number of reported interactions involving Ig/FNIII-like glycoproteins and the recent discovery of novel members of this family however (Connelly et al., 1994; Yoshihara et al., 1994), it is conceivable that axonin-1 ligands other than Nr-CAM might exist on glial cells.

Pretreatment of the dissociated DRG cells with anti-Nr-CAM antibodies also reduced the binding of axonin-1-coated Covaspheres to neurites by  $\sim 20\%$ . This appears to be in contrast to published earlier data (Kuhn et al., 1991), where a complete binding blockage of axonin-1 beads to neurites by anti-Ng-CAM antibodies was reported, suggesting that Ng-CAM is the only neuronal axonin-1 receptor. However, the cells used in the two studies were investigated at different times in culture: in the previous investigation, the DRG cells were cultivated for 5 d so that



**Figure 10.** Antibody perturbation of contacts between neurites and glial cells. Dissociated DRG cells were cultured on laminin in serum-free medium in the presence of different Fab fragments, and the formation of contact sites between neurites and glial cells was visualized by phase-contrast optics (A, C, and D) and scanning EM (B). (A and B) In the absence of antibodies numerous contact sites between neurites and glial cells were observed (arrows in A). These contact sites were identified by the formation of halos in the phase-contrast optics (A). (B) Scanning EM micrograph of a contact site demonstrating the close association between a glial cell and neurites. The number of such contact sites was markedly reduced if the cells were cultured in the presence of anti-axonin-1 (C) or anti-Nr-CAM (D) Fab fragments. Bars, 25  $\mu$ m (in A, C, and D); 10  $\mu$ m (in B).

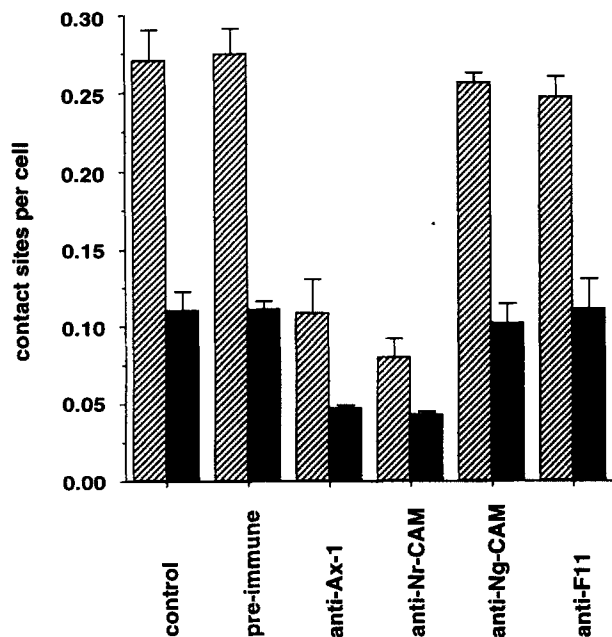
only few nonneuronal cells would be present at the time of analysis (Kuhn et al., 1991), whereas the cultures used in our study were analyzed after 1 d when they contained a considerable number of nonneuronal cells. Therefore the neuritic axonin-1-binding sites that were blockable by anti-Nr-CAM antibodies may be attributable to a higher quantity of Nr-CAM being exposed at the neurite surface in the initial stage of cultivation and/or the presence of Nr-CAM on glial cell membranes ensheathing neurites. Further investigations will be required to determine whether binding between neuritic axonin-1 and Nr-CAM has functional relevance for the interaction between neurites, for example in neurite outgrowth or fasciculation.

The specificity of the interaction between axonin-1 and Nr-CAM was demonstrated using purified axonin-1 and Nr-CAM, as well as several control proteins, covalently coupled to Covaspheres. When axonin-1- and Nr-CAM-coated Covaspheres were incubated together, the percentage of beads found in mixed aggregates was in a similar range to that reported for axonin-1 and Ng-CAM Covaspheres (Kuhn et al., 1991). In accordance with previously reported results (Kuhn et al., 1991), a homophilic aggregation of axonin-1 Covaspheres was not observed, in spite of the demonstrated ability of axonin-1 to mediate cell-cell adhesion by a homophilic interaction if it is heterologously overexpressed by myeloma cells (Rader et al., 1993). In agreement with previously reported findings (Mauro et al., 1992; Morales et al., 1993), Nr-CAM Covaspheres showed homophilic binding properties, but were never found in mixed aggregates with Ng-CAM Covaspheres. In summary therefore, three different interactions of Nr-CAM have been characterized at the molecular level to date: (1) A homophilic binding (Mauro et al., 1992); (2) a heterophilic interaction with F11 (Morales et al., 1993); and (3) a heterophilic interaction with axonin-1 (this study). Similar

to the Nr-CAM/Nr-CAM (Mauro et al., 1992) and F11/Nr-CAM (Morales et al., 1993) interactions, binding between axonin-1 and Nr-CAM Covaspheres was found to be independent of the presence of divalent cations, indicating that the axonin-1/Nr-CAM interaction is distinct from a reported heterophilic, divalent cation-dependent binding activity of Nr-CAM to an unknown component on L cells and primary chicken fibroblasts (Mauro et al., 1992).

#### ***Axonin-1 and Nr-CAM Are Involved in the Formation of Neurite/Glia Contacts in the PNS***

In our studies using dissociated DRG cultures, both anti-axonin-1 and anti-Nr-CAM Fab fragments markedly perturbed the formation of contacts between glial cells and neurites. Obviously, the only single interaction that can be perturbed by both anti-axonin-1 and anti-Nr-CAM antibodies is that between neuritic axonin-1 and glial Nr-CAM. However, both axonin-1 and Nr-CAM have other ligands that could contribute to the formation of neurite/glia contacts. The following results indicate that among the axonin-1 ligands, namely Nr-CAM, Ng-CAM, and axonin-1, only Nr-CAM on the glial cells is a major glial ligand to neuritic axonin-1: (a) Antibody perturbation experiments with anti-Nr-CAM Fab fragments reduced the binding of axonin-1 Covaspheres to glial cells by 70%; (b) a homophilic binding of axonin-1 can be excluded because axonin-1 is not expressed by glial cells; and (c) a heterophilic binding of glial Ng-CAM to neuritic axonin-1 is excluded as a relevant interaction because only a few glial cells express Ng-CAM and because neither the binding of axonin-1 Covaspheres to glial cells nor the formation of neurite/glia contacts was affected by anti-Ng-CAM antibodies. Conversely, all presently known ligands of Nr-CAM, namely axonin-1, F11, and Nr-CAM, are exposed at the



**Figure 11.** Quantification of perturbation of contacts between neurites and glial cells. Dissociated DRG cells were cultivated on laminin in the presence of different Fab fragments against CAMs. For each experimental condition, the total numbers of neurons, nonneuronal cells, and contact sites (glial cells in close contact with neurites) was counted over the same area of the cell culture dishes (for details see Materials and Methods). For normalization, the numbers of contact sites per neuronal (*hatched columns*) and nonneuronal cell (*solid columns*) were calculated. Mean values  $\pm$  SEM for three different experiments are given. These values were markedly reduced if the cells were cultivated in the presence of anti-axonin-1 Fab fragments (by 60 and 57%, respectively) or anti-Nr-CAM Fab fragments (by 70 and 61%, respectively) when compared with the control without antibodies. Cultivation of the cells in the presence of anti-Ng-CAM, anti-F11 or preimmune Fab fragments had no effect on the observed number of contact sites.

surface of DRG neurites. A substantial contribution of binding of neuritic F11 to glial Nr-CAM is excluded, since anti-F11 Fab fragments did not interfere with the formation of neurite/glia contacts. Homophilic binding between neuritic and glial Nr-CAM can not be ruled out by our antibody perturbation studies. However, a 60% reduction in neurite/glia contact sites by anti-axonin-1 antibodies (combined with the facts that Nr-CAM is the only functionally relevant axonin-1 ligand on glial cells and axonin-1 is not present on glial cells) suggests that axonin-1 serves as a major neuritic ligand of glial Nr-CAM in the formation of neurite/glia contacts. Taken together, these data strongly support the occurrence of a direct interaction between neuritic axonin-1 and glial Nr-CAM in the formation of neurite/glia contacts in the PNS.

Several adhesion molecules of different structural classes have been reported to be involved in interactive events between peripheral glial cells and axons during the different stages of myelin formation (for reviews see Doyle and Colman, 1993; Martini, 1994). For example, *N*-cadherin has been demonstrated to further the formation of stable membrane contacts between Schwann cells

and neurites in vitro (Letourneau et al., 1991). Members of the Ig superfamily have been suggested to be involved at various stages of myelination. L1 has been shown to further the extension of processes from Schwann cells between fasciculating neurites (Seilheimer et al., 1989; Wood et al., 1990). The myelin-associated glycoprotein (MAG) has been reported, on the basis of in vitro experiments using cultured rat Schwann cells infected with a retrovirus expressing MAG antisense RNA (Owens and Bunge, 1991), to play a role in the early segregation and ensheathment of large caliber axons. In contrast, very recent gene knock-out studies suggested that MAG is not critical for myelination in the PNS (Li et al., 1994; Montag et al., 1994). Using the same experimental approaches, protein P<sub>0</sub> has been demonstrated to mediate formation of a compact myelin sheath (Owens and Boyd, 1991; Giese et al., 1992). The present report on an interaction between neuritic axonin-1 and glial Nr-CAM during the association between neurites and glial cells provides another event in what may emerge as a complex pattern of parallel and sequential processes essential for myelination. Further work will be required to elucidate the mechanisms by which such interactive events involving adhesion molecules contribute to the development of an intact myelin sheath.

Binding between neuronal axonin-1 and nonneuronal Nr-CAM may also be involved in earlier developmental functions, such as axon guidance. As demonstrated recently by Stoeckli and Landmesser for the projection of commissural axons in the developing spinal cord (Stoeckli and Landmesser, 1995), injection of antibodies against both axonin-1 and Nr-CAM into chicken embryos resulted in pathfinding errors, with many commissural axons failing to cross the midline and turning instead along the ipsilateral floor plate border. These results strongly suggest that Nr-CAM expressed by floor plate cells (Krushel et al., 1993) could act in guiding axonin-1-expressing commissural axons. A guidance function for axonin-1-expressing axons could also conceivably occur at other locations via nonneuronal Nr-CAM. Nr-CAM intriguingly appears on Müller glial end-feet in the developing chicken retina (Kayyem et al., 1992b) at the time when axonin-1-expressing axons of retinal ganglion cells (Ruegg et al., 1989b) grow out. Like other CAMs of the Ig/FNIII-family, axonin-1 and Nr-CAM appear to be involved in various developmental processes in the nervous system. The different functions mediated by Ig/FNIII-glycoproteins may be determined by their spatial and temporal expression pattern and, since these molecules exhibit multiple binding activities, it is conceivable that different interactions may activate different functional properties of these molecules. The aims of our future work therefore, are to carry out a detailed molecular investigation of the mechanisms by which axonin-1 and Nr-CAM bind each other and to determine how this binding affects the interaction of axonin-1 or Nr-CAM with their other ligands and exerts control over their functions.

We thank Eva Niederer for her excellent support of our work with the flow cytometer, Walter Scherle for help with scanning EM, Urs Ziegler for assistance in microscopic analysis, Doris Kemler and Heinz Sonderegger for help with figures, and Gerard Wall for reading the manuscript. Furthermore, we thank E. J. de la Rosa, G. Morales, F. G. Rathjen, N. Ratner, and M. Chiquet for providing antibodies.

This work was supported by grants from the Swiss National Science Foundation, the Schweizerische Multiple Sklerose Gesellschaft, the Fonds für medizinische Forschung der Universität Zürich, and the National Institutes of Health (EY07725).

Received for publication 16 May 1995 and in revised form 17 August 1995.

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